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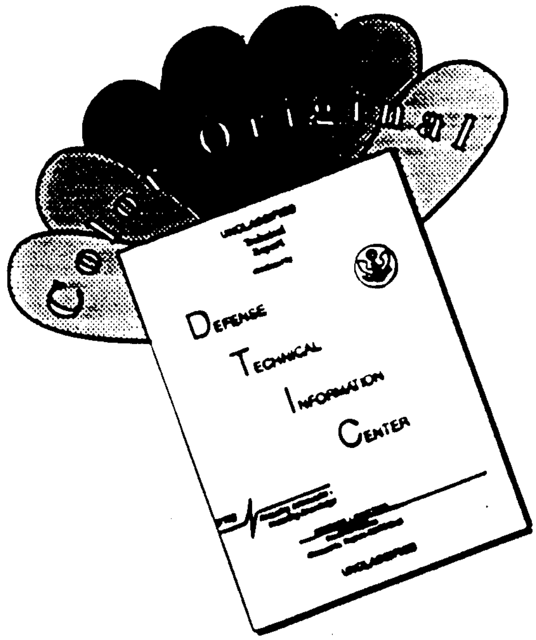

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Our earlier results showed that the presence of cytokeratin positive epithelial cells in the bone marrow is an independent predictor of worse prognosis in human breast cancer. The biological origin and the cellular and molecular characteristics of these cells in not known and was the objective of the present proposal. We have characterized a set of 47 primary breast cancers with respect to expression of mutant p53 and HSP27. Our results are consistent with the findings that expression of mutant p53 is associated with metastatic spread be it occult as found in bone marrow or positive lymph nodes. 57% of p53 eve tumors were associated with bone marrow micrometastasis (BMM) as compared to 15% of p53-ve tumors with BMM positive. We have developed a novel culture technique that can be adapted to separate the epithelial cancer cells in the bone marrow. This will allow us to fully characterize these cells at the cellular and molecular level and define their origin, thus fulfilling the major objective of this proposal.

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ANNUAL REPORT - GRANT #DAMD 17-94-J4471

Introduction

The presence of micrometastatic cells in the bone marrow is associated with poor prognosis and early relapse. These patients may be candidates for adjuvant therapy. However, the presence of epithelial cells alone is not enough to justify the use of adjuvant therapy unless the cellular and molecular characteristics of these cells is established. It is the aim of the proposal to determine the cellular and molecular nature of these cells and define their origin. Characterization of these occult metastatic cells in large number to determine their biological characteristics is not feasible by pathological examination alone and it is apparent that isolation and molecular characterization of these cells is crucial. Using the reverse transcriptase polymerase chain reaction it is possible to define the molecular parameters that can genotype these cancer cells. We have developed a novel method to isolate these cells for growth in culture. Both of these research methods, though exclusive of each other, have to be carried out simultaneously. Our research efforts were directed to :

A. Define the molecular characteristics of these cells with respect to parameters that define primary breast cancer in humans.

B. Isolate these cells and grow them in culture so that their tumorigenic potential can be assessed.

Nature of the Problem

No single marker fully characterizes human breast cancer. Several oncogenes, p53, HER-2/neu, ras, myc, have been implicated in human breast cancer but only serve to define subsets of tumor cells. We attempted to investigate the role of mutant p53 in primary human breast cancer and its association with bone marrow micrometastasis.

The difficulty in growing the epithelial cells for the bone marrow is as old as cell culture itself and the problem of growing epithelial cells from human primary breast is well recognized. Human breast cancer epithelial cells have a tendency not to grow in isolation thus compounding the problem of growing scarce epithelial cells from the marrow. However, we have devised a novel technique by which we can separate the cancer cells. This enables us to get purified population of cells which can be characterized at the molecular and cellular level. The tumorogenic potential of these cells can be determined provided large numbers of these cells can be grown in culture. It is not known whether these cells, once adapted in culture, would have the same in vivo characteristics.

Background of previous work

Ninety-five percent of patients who present with breast cancer apparently have local disease without evidence of distant metastatic spread on pretreatment staging by conventional methods (1). Despite improvements in surgical techniques, radiotherapy and drug treatment, one third of all patients relapse and die within ten years, and this proportion has not changed significantly. It is accepted that this group of patients has micrometastatic disease at presentation that cannot be detected by current standard methods (2-5).

The skeleton is the most common site of distant metastases for breast cancer (6) and is frequently the first site at which distant metastasis is detected. The concept of investigating bone marrow as a site for occult micrometastases has validity from two aspects. First, bone metastases start from bone marrow invasion (7) and second, the lymphovascular function of the marrow represents an ideal location to detect transient cancer cells. Conventional techniques of examining bone marrow have a very small likelihood of identifying tumor cells at the time of initial treatment (8-11).

Immunocytochemical Examination of Bone Marrow

A group at the Ludwig Institute for Cancer Research (LICR) in London examined bone marrow aspirates using antisera prepared against human epithelial milk-fat-globule membranes. This antigen was termed the epithelial membrane antigen (EMA) (12). Using an indirect immunoperoxidase technique, it was initially shown that EMA has a widespread but highly selective distribution in human tissues. EMA staining was observed in normal breast epithelium, primary mammary carcinomas, carcinoma cells infiltrating bone marrow, xenografts of primary carcinoma in nude mice and the MCF-7 cell line (12). Subsequent more detailed studies of breast cancers showed strong staining (13), and single metastatic breast tumor cells in bone marrow showed intense staining. Bone marrow aspirates from 20 disease-free patients treated for breast cancer 3-5 years previously were negative for EMA stained cells. An additional eight patients with positive nodes were also EMA negative. Eight of 43 (18.6%) patients with metastatic disease and negative routine marrow histology had EMA positive cells in the marrow, and it was concluded that the sampling of paraffin-embedded sections used in this study may be less satisfactory than smears (14). Continued studies using aspiration smears were more satisfactory (15-16. Early data also suggested that multiple sites yielded more information than a single site of bone marrow aspiration (17). The presence of EMA stained cells in the marrow correlated with some of the standard prognostic indicators (e.g.: tumor diameter, vascular invasion, positive nodes) (18,19). The results in 307 patients with primary breast cancer showed that 26.4% had EMA-positive cells in the bone marrow at the time of diagnosis (20). At a median duration of follow-up of 28 months, 75 patients had relapsed (60 with distant disease). Of the 60 patients with distant disease, 43% had EMA-positive cells at initial diagnosis. The presence of EMA-positive cells predicted for bone metastasis, and the authors concluded that this technique may be of help in selecting patients at risk who could benefit from systemic therapy.

Other investigators have confirmed the observation of immunoreactive cells, using either EMA or anti-cytokeratin (AE1) antibodies in the bone marrow of early stage breast cancer patients (21,22). An update of the Munich group's early results suggests a high subsequent relapse rate (80%) of breast cancer patients with immunoreactive cells observed in the bone marrow at the time of initial treatment (23). A highly sensitive immunoflourescent monoclonal antibody method has been developed and used for preliminary studies (24-27). The key to the detection and characterization of cells has to be evaluated in relation to standard pathologic prognostic indicators such as tumor size, grade, lymphatic and vascular invasion and lymph node status.

Several mouse monoclonal antibodies specific for epithelial cells have been developed in our laboratory. These antibodies (C26, T16), along with a commercially available monoclonal antibody specific for epithelial cells (anti-cytokeratin intermediate filament antibody AE1, Labsystems, Finland) react with distinct epithelial-specific antigens. All are epithelial-specific and each reacts with most breast cancers tested (28-33). These monoclonal antibodies have not been shown to react with normal marrow components.

Preliminary Results from our Previous Study

The results of 348 patients are available and the observed prevalence of bone marrow micrometastases (BMM) was 32%. Standard perioperative staging tests and light microscopy of the bone marrow did not show any evidence of metastatic disease. There was no significant difference in the frequency of BMM in patients with maximum tumor diameter ≤ 2 cm when compared to hosts with ≥ 2 cm diameter. Similarly there was no difference in frequency of BMM in patients with axillary lymph node metastases when compared to those with negative lymph nodes. Both the prevalence and number of tumor cells were statistically independent of tumor diameter and lymph node status. At a median follow up of 2.5 years the proportion relapse free is related to the bone marrow status and the number of tumor cells detected. Presence of bone marrow micrometastases showed a statistically significant increase in early relapse ($p=0.05$).

Our data show that bone marrow micrometastasis in patients with breast cancer at the time of initial diagnosis is a useful parameter that can predict early relapse, and enhance the utility of existing standard prognosticators. Patients with epithelial cells in the bone marrow clearly have a higher risk of recurrence and as such it may be essential aggressive methods to cure residual disease (34,35), however the presence of epithelial cells alone is not enough to justify the use of adjuvant therapy unless the nature and characteristics of these cells are established. This proposal is focussed to define the relationship of the epithelial cells in the marrow with the primary breast cancer.

Purpose of the Present Work

The overall purpose of the present work is to characterize the cytokeratin positive epithelial cells in the bone marrow at the cellular and molecular level. Experiments designed and executed during the current year meet this objective. We have examined: 1) expression of mutant

p53 in the primary tumor and have correlated expression of this oncogene with prevalence of BMM, 2) methods to isolate and culture cytokeratin positive epithelial cells in the bone marrow using novel separation techniques.

Method of Approach

The molecular characterization of epithelial cells in the bone marrow is germane to our proposal, but before this is attempted, correlative evidence to link the parameters that we intend to study is crucial. We have tried to link one such parameter namely the expression of mutant p53 in the primary tumor. Mutant p53 expression was determined by immunohistochemistry (IH) and by reverse transcriptase PCR (RT-PCR). We also included expression of heat shock problem 27 as another prognostic factor that could determine specificity in our correlation association of expression of prognosticators with prevalence of BMM.

For isolation and growth of epithelial cells from bone marrow of patients, we have used a hydrophobic polymer polyvinylidine flouride (PVAF) a chemically stable but biologically inert polymer. Cellswere grown in polymer tubes and then examined microscopically by cutting the polymer tubes into sections and fixing and staining the attached cells on one or more sections.

Body

The overall objective of this study is to delineate the molecular and cellular characteristic of cytokeratin positive epithelial cells in the bone marrow of breast cancer patients. The presence of these cells has been associated with worse prognosis and an early relapse. Experiments conducted during the past year focussed on:

1) Determining the molecular parameters in the primary tumor and its association with prevalence of bone marrow micrometastasis. Expression of mutant p53 and HSP27 in primary breast cancer was examined by immunohistochemistry and RT-PCR and was correlated with BMM. These studies were done with an objective to define the molecular parameters that would be best suited to be used to characterize bone marrow positive epithelial cells considering the limitation of sample size that is involved in such analyses.

2) Determining the condition of isolation and culture of epithelial cells from bone marrow. This procedure was attempted because the success of these experiments will enable us to better characterize these "occult" cancer cells at the cellular level and possible determine their tumorogenic potential.

We will describe the results of experimental approaches and conclusion of both of these aims separately.

Expression of Mutant p53

Mutations of p53 have been widely described in all human cancers including breast (36,37). p53 is a tumor suppressor gene rarely detected, as its protein product is unstable. Mutation in the gene confers stability and as such can be detected at the RNA and protein level. Mutant p53 cooperates with 'ras' to transform rodent fibroblasts (38-40) while murine wild type p53 suppresses 'ras' plus mutant p53, or myc-induced transformation (41). Mutated p53 has been found to be present in many breast cancer patients and the mutation has been preserved through tumor progression. The mutations are widespread and span exons 5 to 8. The mutations found in the primary cancer are the same as those seen in metastatic lymph nodes (42,43). These results suggest that p53 product either contributes to the maintenance of tumorigenic phenotype or promotes aggressive behavior and may be an important contributor to the tumor ability to early metastasis.

Analysis of forty seven primary breast tumors frozen in OCT was analyzed for the expression of mutant p53 and HSP27. Adjacent expression of the oncogene and an important breast cancer prognosticator was done by immunohistochemistry which was confirmed by RT-PCR. Expression of p53 and HSP27 was correlated to the presence of bone marrow micrometastasis.

Method Used

1. Immunocytochemical Staining for p53

Immunohistochemical staining procedures are similar to the ones described in earlier publications (42,44,45). Briefly, the smears are brought to room temperature and washed in buffered saline. Immunohistochemistry using Ab-6 mouse monoclonal antibody, (1:100 dilution, Oncogene Science, USA.) for p53; an antibody to HSP27 was obtained from Neomarkers (California) are used. Briefly the antiserum (in respective dilution) is applied on 8μm sections, incubated at room temperature. Incubation with secondary antibody is followed by avidin-biotin immunoperoxidase staining with diaminobenzidine as chromogen. The slides are counterstained with hematoxylin.

2. Isolation of RNA and PCR Amplification

Total RNA is isolated using RNAzol (Biotecx) solution. Briefly, the slides are overlayered with 500 μl of RNAzol and incubated on ice for 15 minutes. The solution is carefully collected and transferred into an eppendorf tube and the slide further washed with an additional 500 μl of RNAzol to ensure complete transfer. 0.2 ml of chloroform is then added, incubated for 15 minutes at 4° C, vortexed ad the mixture centrifuged at 12000Xg for 15 minutes. the RNA is collected from the aqueous phase and the RNA precipitated by an equal volume of isoproponol. The RNA is dissolved in nucleic acid free water, incubated with RQ Dnase I (0.1 U/μl) and Raasin (2 U/μl) and then purified by phenol:chloroform extraction and reprecipitated by 2

volumes of ethanol. The purified RNA is used for cDNA synthesis. First strand cDNA synthesis and subsequent amplification is done using a kit from Perkin-Elmer Cetus. The method utilizes rTth DNA polymerase and the advantage is that cDNA synthesis and amplification for a number of genes is done in a single tube. PCR amplification is done at 95° C for 1 minute 30 seconds - 55° C for 1 minute for 45 cycles and further polymerized at 72° C for 10 minutes and soaked at 4° C. The amplified products are analyzed on 3% NuSieve agarose containing 1% SeaKem agarose. The primers used to amplify a 7126p fragment of mutant p53 spanning exous 4 to 10 were:

p53 Sense 5' GGGACAGCCAAGTCTGTGACT 3', Antisense 5' CCTGGGCATCCTTGAGTT 3' (37).

Results and Conclusions

1) Mutant p53 was expressed in seven of the forty seven primary tumors analyzed. Expression as determined by immunohistochemistry was found to be intraductal as well as infiltrating. A representative of infiltrating (Figure 1) and intraductal p53 expression (Figure 2) is attached. Complete concordance was found with RT-PCR and immunohistochemistry analysis (Figure 3).

2) Correlative studies were performed with prevalence of bone marrow micrometastasis and p53 expression. Four of the seven p53 positive tumors were BMM positive (57%) and six of the forty p53 negative tumors were BMM positive (15%). This difference is statistically significant (p=0.02). Three of seven p53 positive tumors had lymph node metastases. HSP27 was localized in infiltrating (35%) and intraductal (35%). No correlation was found either with BMM or mutant p53. Representative expression of HSP27 intraductal and infiltrating is presented in Figures 4 and 5.

It is clear from the above results expressing mutant p53 is associated with an aggressive subset of breast cancer cells with propensity to metastasis as all of the seven samples that were found to be positive for p53 expression had metastatic spread in some form or the other. They were either BMM positive or had positive lymph nodes. This however was not true in the case of HSP27.

Future studies will include the examination of specific mutation in p53 and to determine if the mutation is conserved. It can be concluded from this set of results that mutant p53 may be an important parameter in study cytokeratin positive epithelial cells in the marrow and their mutations characterized. This may help define the origin of these cells.

<u>In vitro</u> **culture of epithelial cells in the marrow**

Objective

To separate and culture micrometastatic epithelial cells found in the bone marrow of some breast cancer patients.

Experimental Design

We have established a new approach in cell culture technique which shows promise in helping to achieve the set objective. It also shows promise for examining bone marrow for features that may have diagnostic value. The new technique has stimulated the cells to unusual patterns of growth and proliferation. Instead of cell culture flasks or wells with artificial cell attachment coating, we are using microporous polymer membrane tubes as cell culture containers. The tubes are positioned vertically so that oxygen can diffuse directly to the attachment site of the cells. The hydrophobic nature of the polymer prevents the media from seeping out through the pores. Additionally, the microporous structure of the membrane surface is a particularly desirable feature for culturing most cells and particularly epithelial cells. The hydrophobic polymer used in this work, polyvinylidene difluoride (PVDF), is chemically stable and biologically inert. The tubes are charged with media and the cells are seeded into the tubes and permitted to sediment to the bottom. Cell cultures are examined microscopically by cutting the polymer tubes into sections and fixing and staining the attached cells on one or more section. Cell colonies can be 'passed' for further culturing by inserting into fresh polymer tubes cut segments of tubes with attached cells. This method avoids subjecting the cells to the trauma of trypsin treatment.

Results and Conclusion

New Pattern of Cell Growth and Proliferation

In using our type of cell culture container we have encountered an unusual pattern of cell proliferation. The cells, for example MCF-7 cells or some nucleated bone marrow cells, in addition to the usual compact colonies form also extended sparsely populated or even empty appearing extracellular matrix (ECM) structures which in further periods are progressively filled with cells by proliferation and perhaps by migration of cells into the domain of these matrix structures. Because of their appearance we have termed the newly observed matrices, gauze matrices.

Gauze matrix formation is induced by the direct oxygen supply to the cell attachment site. Reducing oxygen access to the cell attachment site by reducing polymer membrane gas permeability or by obstructing gas flow to the external surface of the membrane,eliminates gauze-matrix formation. Gauze-matrix forms principally on the vertical walls of the polymer tube and more densely in the higher zone where the membrane wall is thinner and thereby gas permeability more rapid; it rarely forms on the bottom of the culture tube. There is photographic evidence that the gauze matrix is formed by the trail left on the polymer surface by migrating cells. The migrating cells leave behind a trail of translucent material attached firmly to the surface of the microporous membrane. Accumulation of these trails forms a matrix, very often in the form of a

ribbon of trails, but other patterns of trail distribution are also seen. Extensive library of microscope photographs suggests that these matrices may be sacks and that the cells are confined within these sacks. The gauze matrix colony is very likely a three dimensional structure. The cells residing within these gauze matrices, in contrast to the compact colonies, are not in a cell-cell contact pattern, they are mostly individually separate. In fact, the cells appear three-dimensional. Cells on the vertical walls proliferate almost exclusively within the confines of the gauze matrices.

Formation of gauze matrices is an ongoing process. There are coexisting gauzes in various stages of development. Compact cell colonies form on the bottom of the polymer tube. They die on reaching regional confluence or even before. By contrast, cells in a gauze-matrix survive well in long term cultures. Gauze matrices do not appear to spread to the entire available membrane surface. The pattern and location of gauze matrix formation may be influenced by materials secreted by the cells and absorbed on the membrane in particular locations. Gauze matrices are not found in cell cultures contained in standard commercial cell culture flasks or multi-well trays.

Cell Morphology

As mentioned earlier, in a compact colony all cells have the same appearance and they contact each other. By contrast, in a gauze-matrix environment cells at different stages of growth can be found in close proximity, cell-cell contact is not the rule, and the cells appear to have three dimensional morphology. The unique features of the gauze matrix and the cells within it suggests that the functioning of the cells and their secretions, may possibly be different from those of the same cells in compact colonies. The cells in the gauze matrix may represent more closely cells in the natural environment.

Differences Between Cell Lines

MCF-7 cells form gauze matrices within 24 hours of seeding. Compact colonies develop within 48 hours and they begin to die off within a week. Cells within gauze matrices are maintained in the same tube in a viable state for at least 28 days, even in the absence of fetal bovine serum (FBS) in the culture media. Nucleated bone marrow cells (NBMC, from patients with breast cancer, form gauze matrices with patterns different from those produced by MCF-7 cells. The appearance of the cells within the gauze matrices may differ markedly between bone marrow samples. A variety of cells, lymphocytes for example, proliferates outside the gauze matrices. NBMC cultures were maintained in the presence of either FBS or bone marrow liquid (BML) in the culture media, and marked differences in the culture were observed depending on which component was used. Peripheral blood lymphocytes, from human blood samples, start dying within 24 hours in the absence of serum addition in the culture media. Peripheral blood lymphocytes (PBL), mixed into a MCF-7 cell culture, begin to die within 24 hours in the absence of FBS while the MCF-7 cells form gauze matrices and remain fully viable within them.

Significance

Selective Cell Culturing

The formation of gauze matrices offers approaches to selective cell culturing and purification of a cell line. As described earlier for the case of MCF-7 plus PBL mixed cell culture, the removal of PBS from the culture medium destroys the PBL while the MCF-7 cells remain viable within the gauze matrix. This culture may serve as a model for selective culturing of cells in the bone marrow by manipulating the serum additive to the culture media. Cells come equipped with attachment site receptors, integrins, for the matrices they create. Cancer cells are believed to use these receptor-matrix interactions as migration aids and as stimuli for proliferation. (46) Naturally generated matrices are biologically active materials. They serve not only as attachment sites for the cells but also as reservoirs for various control factors such as growth factors. The gauze matrix, which is a naturally generated matrix, must have attachment sites specific for the cell type that generated it. Also, as stated above, it may have factors, such as growth factors, specific for the cell type. Such features, if sufficiently exclusive, will provide for a purified cell line within the confines of the gauze matrix. What is unique about the gauze matrices is that they are generated as structures initially sparsely populated by cells, with much available space for new, proliferating cell attachment. This certainly suggests that the cells that eventually fill the gauze matrix are a pure line. Artificial attachment matrices are unlikely to offer such cell line purification possibilities.

Diagnostics

Features of bone marrow cultures in the microporous polymer tubes may have diagnostic value. Bone marrow samples produce patterns of gauze matrices and cell culture growth which appear to differ from sample to sample. These differences may be diagnostically meaningful. Cell population within the gauze matrices differs in appearance between bone marrow samples. This too may have diagnostic value. Since cells within the gauze matrices can be maintained and can proliferate in the absence of added serum, this provides an opportunity to examine cell secretions in the culture without the contaminating interference of serum proteins.

Current Work

The immediate tasks are:

Identifying the material of the matrix in MCF-7 and in bone marrow cultures. We are focusing on the major glycoproteins: collagen, fibronectin, laminin, tenascin. Identifying the cells in bone marrow cultures which form matrices and proliferate within them. We are testing first specifically for the presence of epithelial cells. Culturing bone marrow cells under several conditions in the absence of serum to isolate cell lines, and to analyze cell secretions. Establishing a catalog of criteria to describe the various matrix colonies forming in bone marrow cultures. This may be of future diagnostic value. In our current work we are also considering the possibility of emptying a gauze matrix of MCF-7 cells by milk trypsinization, and then using the empty matrix as specific attachment region for epithelial cells present in bone marrow.

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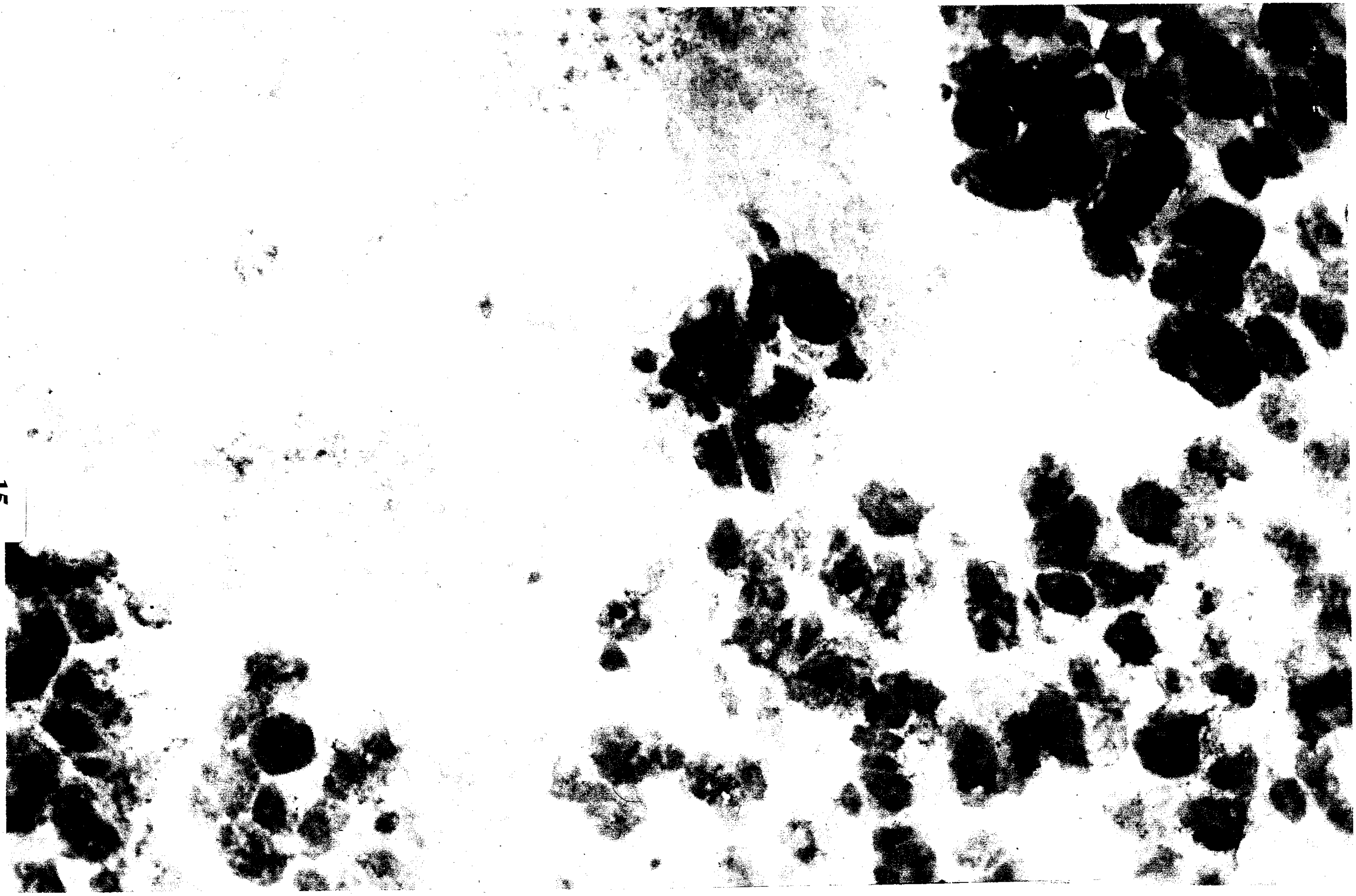
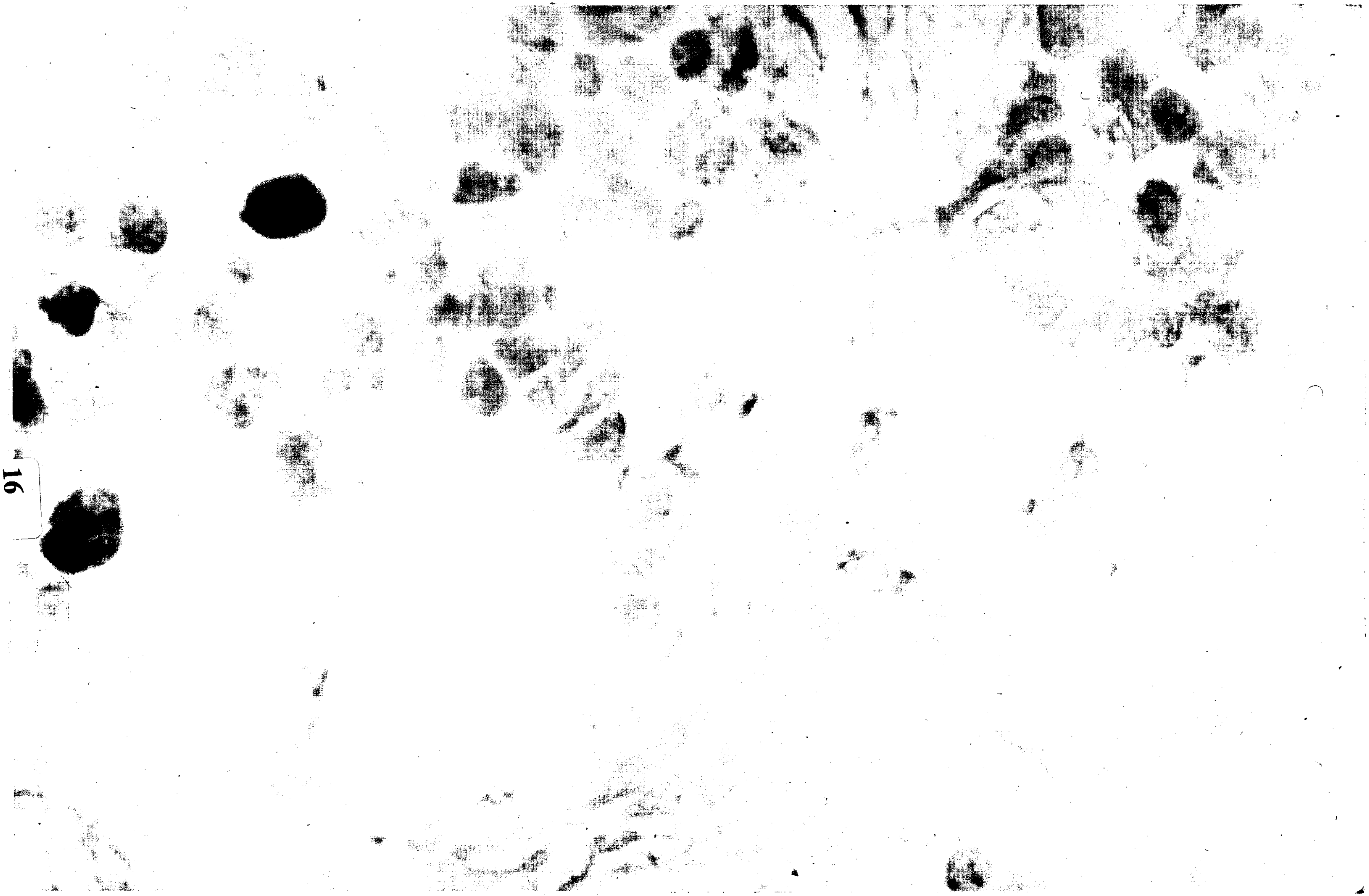


p53: Infiltrating

Fig 1



p53: Intraductal

Fig 2

AMPLIFICATION OF MUTANT p53 by RT-PCR

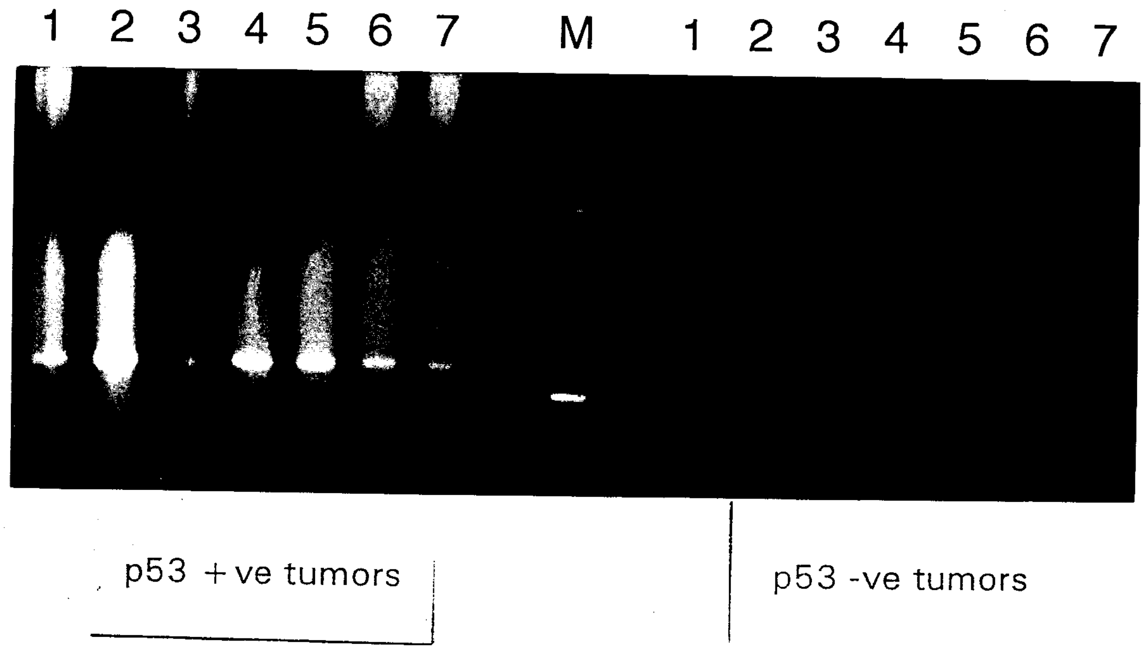


Fig 3

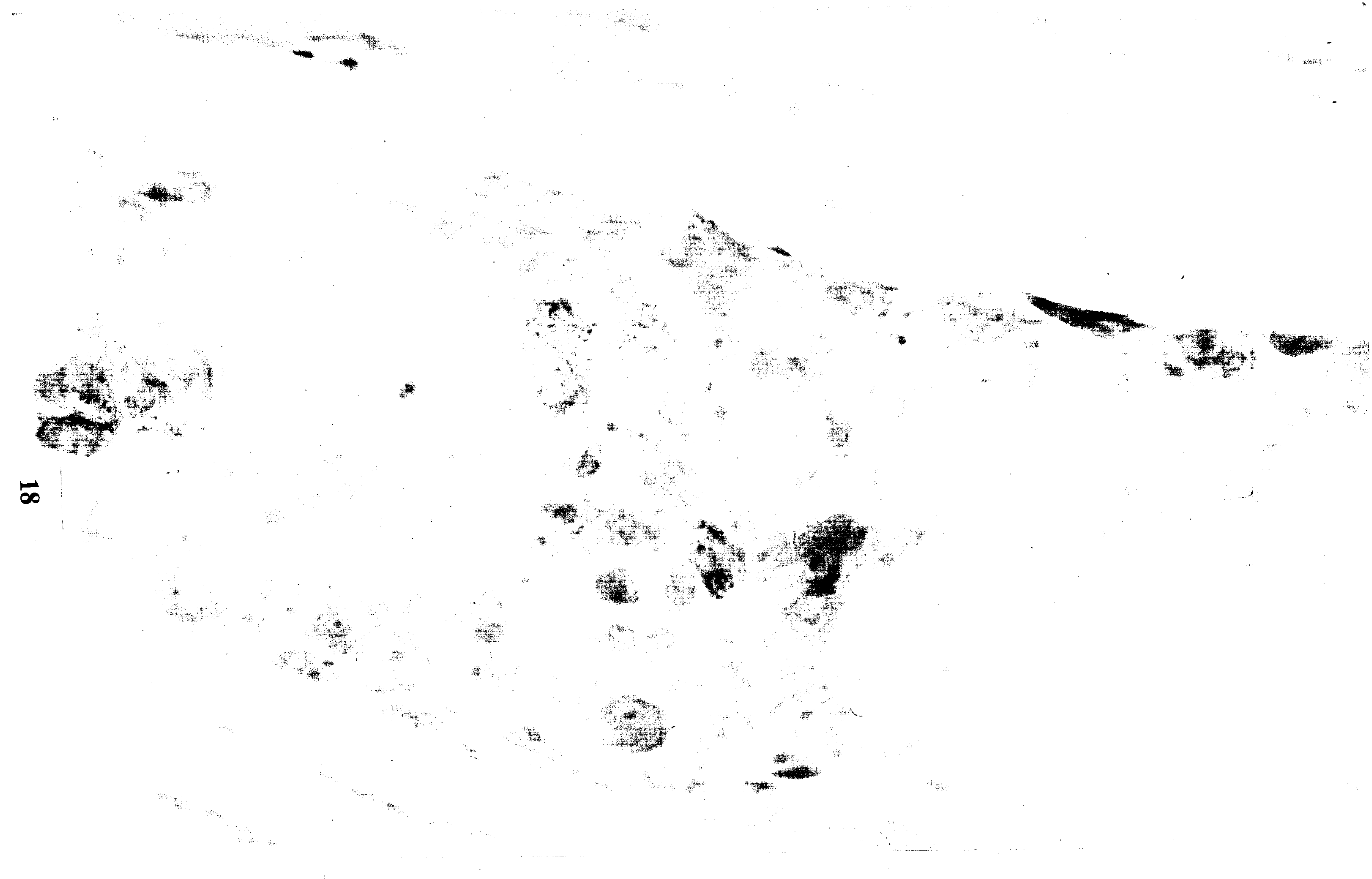


Fig 4

HSP 27: Intraductal

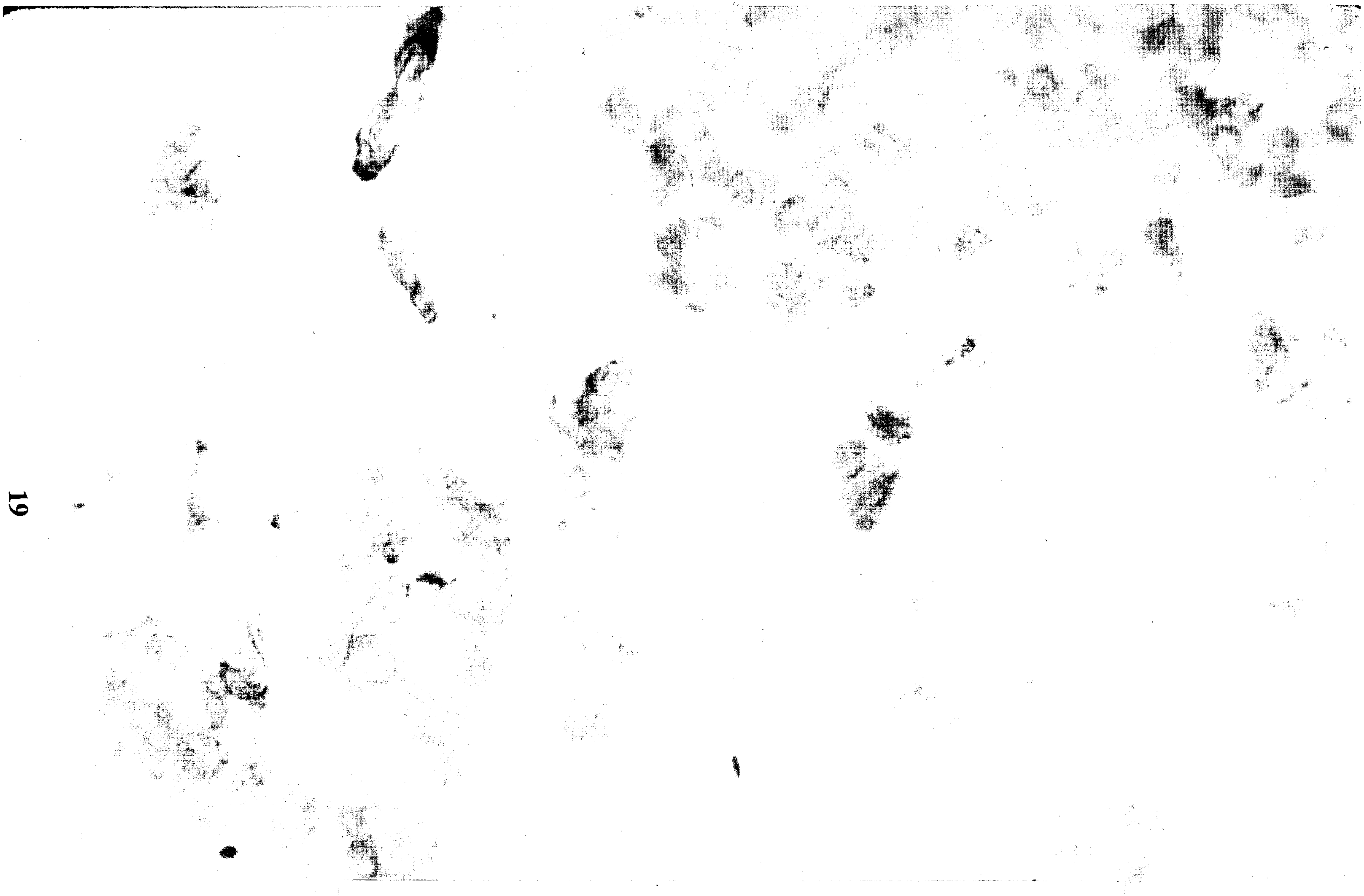


Fig 5

HSP 27: Infiltrating

FIG. 6

CULTURE SYSTEM.

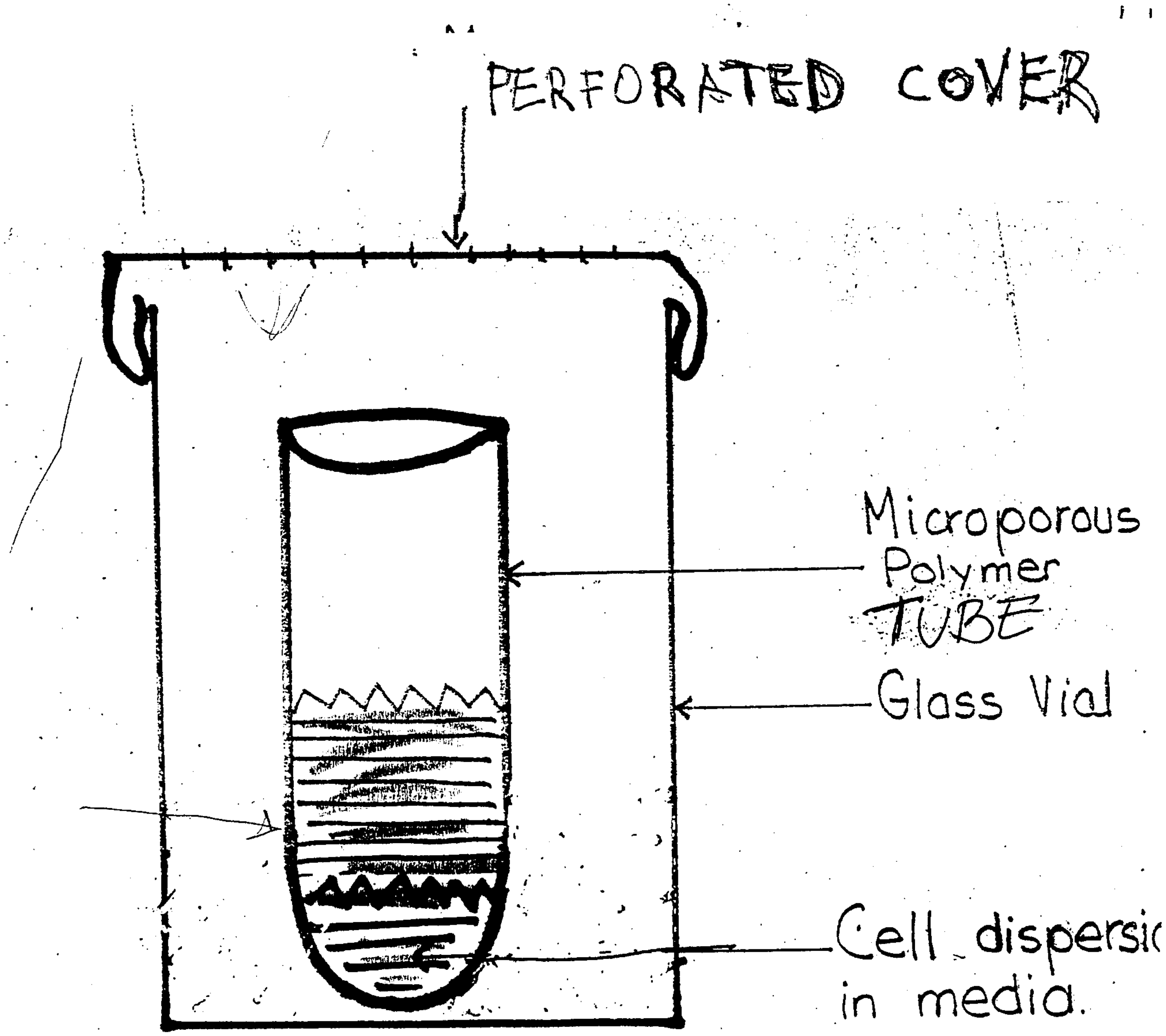


Figure 7

MCF-7 CELL CULTURE

COMPACT COLONY

Magnification ×125

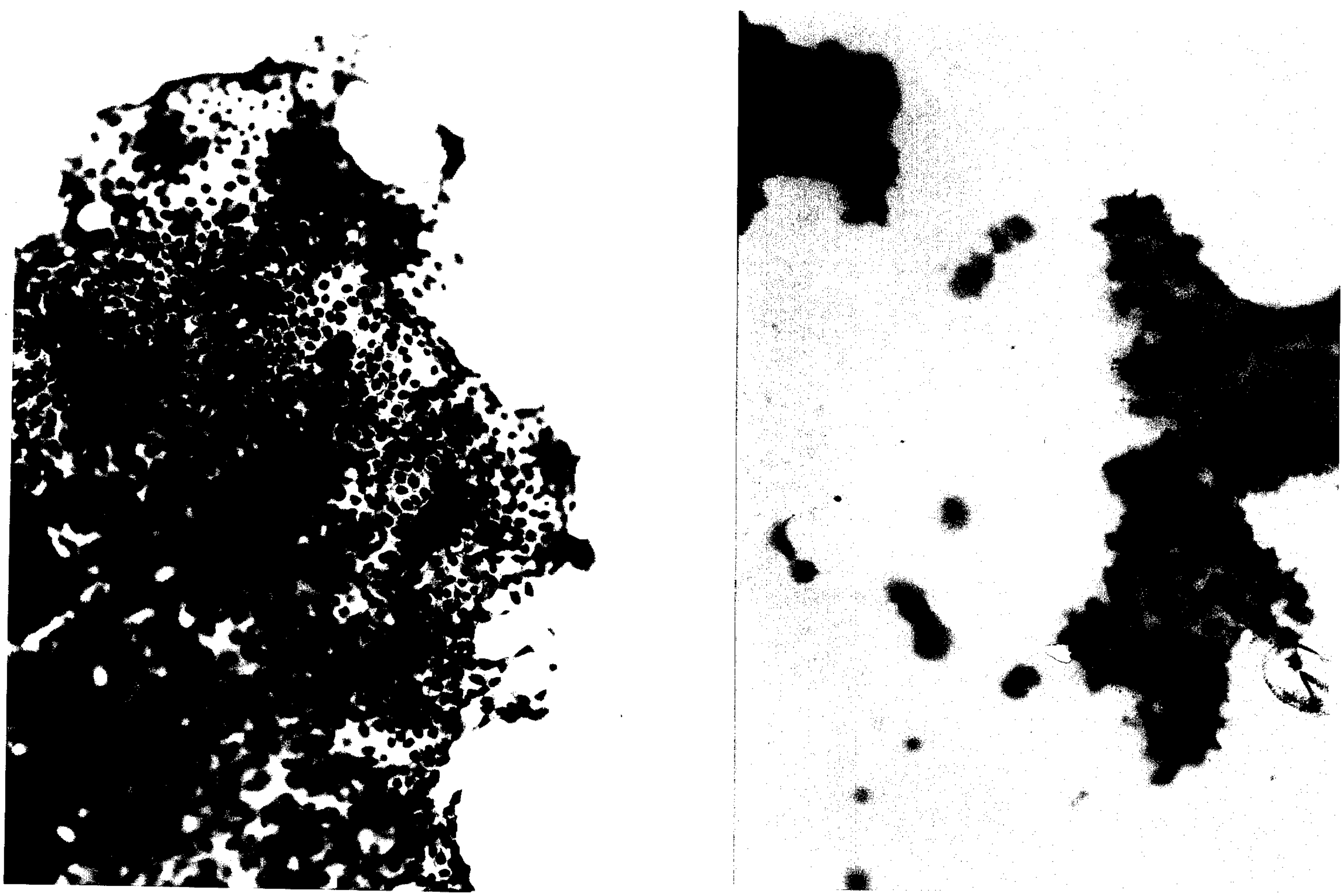


Figure 8

MCF-7 CELL CULTURE

GAUZE MATRIX COLONY

Note absence of cells outside the confines of the gauze matrix. Note the separation of individual cells and the three-dimentional appearance of the matrix.

Magnification ×125

Magnification ×500

Figure 9

MCF-7 CELL CULTURE

GAUZE MATRIX GENESIS (A)

Note regions of matrix formation and the empiness of newly formed matrices

Magnification ×50 *Magnification ×50*

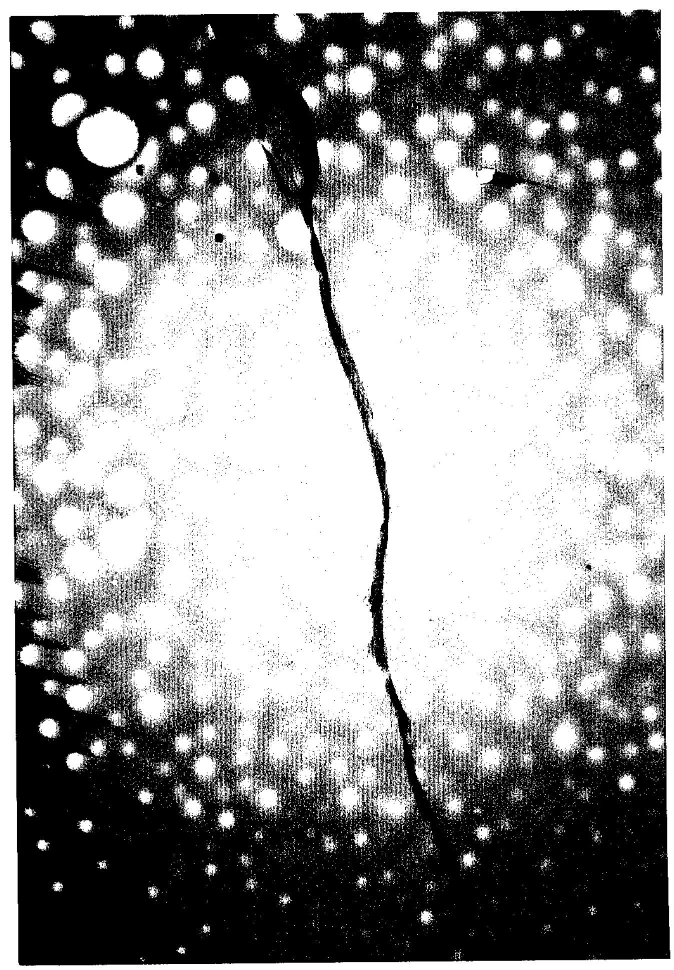


Figure 10

MCF-7 CELL CULTURE

GAUZE MATRIX GENESIS (B)

Contrast of mature and newly formed matrices

Magnification ×125

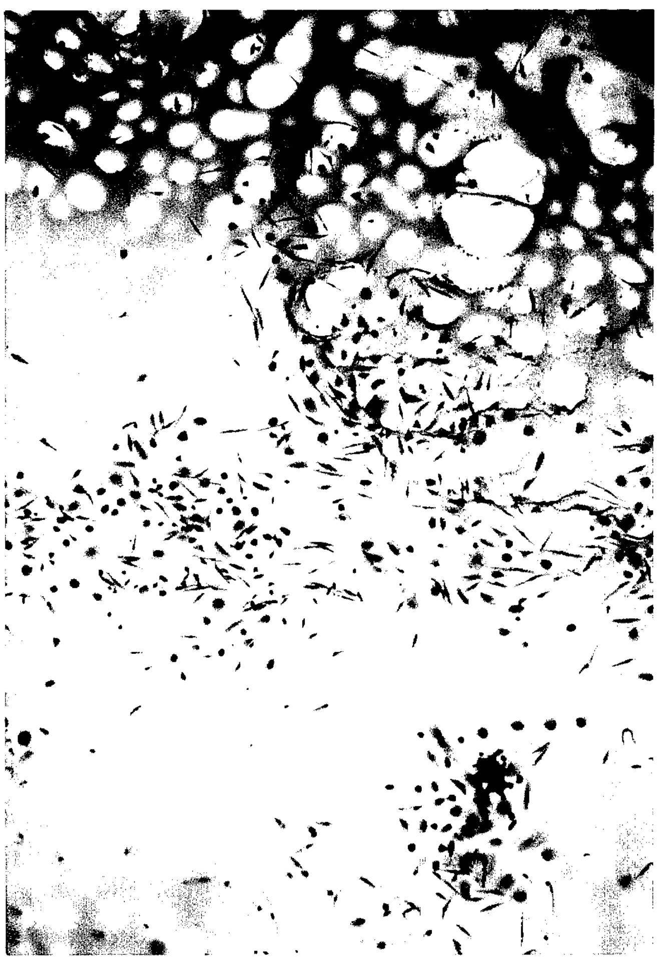
Magnification ×125

Figure 11

PERIFERAL BLOOD (HUMAN) LYMPHOCYTE CULTURE

Note absence of gauze matrices. Lymphocytes are membrane attached.

Magnification ×125



Magnification ×500

Figure 12

BONE MARROW CELL CULTURE

(BREAST CANCER PATIENT)

GAUZE MATRIX COLONY

Note spectrum of cell shapes in the matrix

Magnification ×125

Magnification ×500

Figure 13

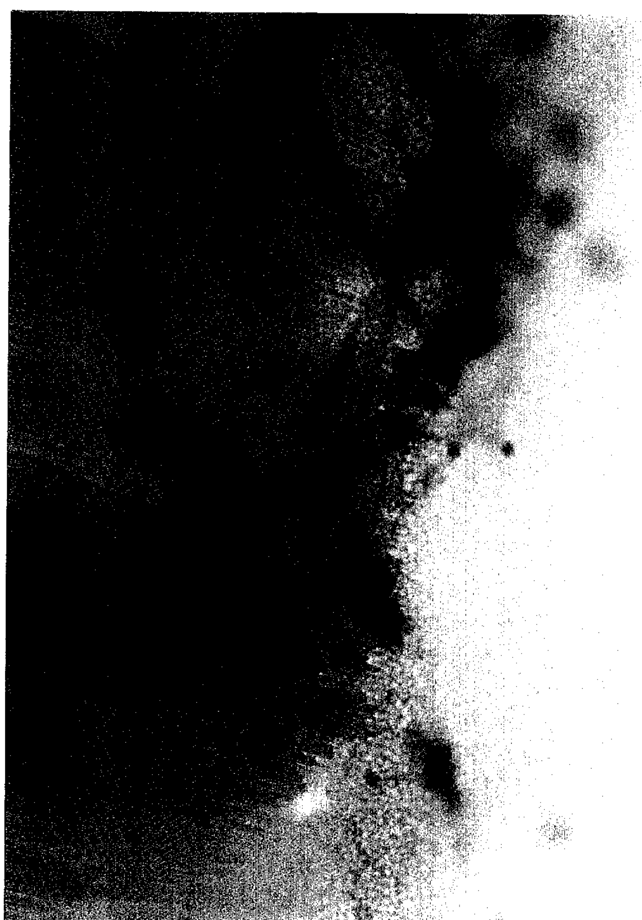
BONE MARROW CELL CULTURE

(BREAST CANCER PATIENT)

GAUZE MATRIX COLONY

Note tenuous structure of the gauze

Magnification ×500



Magnification ×500

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